

RESEARCH ARTICLE

# Purinergic and Cholinergic Drugs Mediate Hyperventilation in Zebrafish: Evidence from a Novel Chemical Screen

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## Abstract

A rapid test to identify drugs that affect autonomic responses to hypoxia holds therapeutic and ecologic value. The zebrafish (*Danio rerio*) is a convenient animal model for investigating peripheral O<sub>2</sub> chemoreceptors and respiratory reflexes in vertebrates; however, the neurotransmitters and receptors involved in this process are not adequately defined. The goals of the present study were to demonstrate purinergic and cholinergic control of the hyperventilatory response to hypoxia in zebrafish, and to develop a procedure for screening of neurochemicals that affect respiration. Zebrafish larvae were screened in multi-well plates for sensitivity to the cholinergic receptor agonist, nicotine, and antagonist, atropine; and to the purinergic receptor antagonists, suramin and A-317491. Nicotine increased ventilation frequency ( $f_V$ ) maximally at 100  $\mu$ M (EC<sub>50</sub> = 24.5  $\mu$ M). Hypoxia elevated  $f_V$  from 93.8 to 145.3 breaths min<sup>-1</sup>. Atropine reduced the hypoxic response only at 100  $\mu$ M. Suramin and A-317491 maximally reduced  $f_V$  at 50  $\mu$ M (EC<sub>50</sub> = 30.4 and 10.8  $\mu$ M) and abolished the hyperventilatory response to hypoxia. Purinergic P2X3 receptors were identified in neurons and O<sub>2</sub>-chemosensory neuroepithelial cells of the gills using immunohistochemistry and confocal microscopy. These studies suggest a role for purinergic and nicotinic receptors in O<sub>2</sub> sensing in fish and implicate ATP and acetylcholine in excitatory neurotransmission, as in the mammalian carotid body. We demonstrate a rapid approach for screening neuroactive chemicals in zebrafish with implications for respiratory medicine and carotid body disease in humans; as well as for preservation of aquatic ecosystems.

## Introduction

The ability to maintain internal oxygen (O<sub>2</sub>) levels within an optimal physiological range is essential for development and survival in vertebrates as this facilitates O<sub>2</sub> delivery to cells and matching of metabolic demands. Vertebrates have respiratory chemoreceptors—specialized cells that sense changes in O<sub>2</sub> partial pressure (P<sub>O<sub>2</sub></sub>)—which elicit compensatory cardioventilatory adjustments during periods of hypoxia. In mammals, the main peripheral chemoreceptors are type I cells of the carotid body, which store neurotransmitters that are released into a chemical synapse during chemoexcitation [1–5]. Although controversial, there is compelling

evidence that the primary excitatory neurotransmitters, which activate afferent nerve terminals in the carotid body, include acetylcholine (ACh) and 5'-adenosine triphosphate (ATP) [6–12]. ACh and ATP activate nicotinic and purinergic P2X receptors, respectively, both of which are non-selective cation channels that mediate excitation.

Homologous to carotid body type I cells are the neuroepithelial cells (NECs) in the gills of teleost fish [13–16]. These cells respond to a decrease in  $P_{O_2}$  with  $K^+$  channel inhibition and membrane depolarization [17,18]; and NECs are believed to initiate reflex responses to hypoxia, such as hyperventilation, increased ventilatory amplitude and bradycardia [15,16,19,20]. It is predicted that during hypoxic stimulation NECs release neurotransmitters into a chemical synapse that activate afferent nerve fibres within the gill filaments. NECs contain synaptic vesicles [13,14] endowed with neurotransmitters, including serotonin (5-hydroxytryptamine, 5-HT), acetylcholine (ACh) and nitric oxide [16,21–24]; and NECs undergo dynamic changes in intracellular  $Ca^{2+}$  upon stimulation [25–27]. However, unlike the carotid body, little is known about cholinergic activity in the fish gill; and there is no functional evidence for purinergic receptors in  $O_2$  sensing in fish.

We hypothesized that exogenous application of neurochemicals that target purinergic and cholinergic receptors accordingly modify the hyperventilatory response to hypoxia via chemosensory pathways in the gills. Zebrafish were used as a model organism as they are a convenient size for recording changes in ventilatory frequency, and rapidly develop a mature  $O_2$ -chemosensory system in the gills during the first two weeks of development [23,28]. We demonstrate purinergic and cholinergic control of the hyperventilatory response to hypoxia in zebrafish, thereby highlighting the evolutionary significance of these mechanisms in  $O_2$  sensing in vertebrates. Numerous studies have used zebrafish to develop *in vivo* chemical screens for drug discovery [29,30]. As an additional objective, we have generated a rapid procedure for the screening of chemicals that may be studied further for their potential as therapeutic agents for respiratory disorders in humans; and for the identification of pharmaceutical or industrial products that threaten aquatic ecosystems.

## Materials and Methods

### Ethical statement

Handling and care of animals were conducted in accordance with the Canadian Council on Animal Care (CCAC) and all protocols were approved by the Animal Care Committee at the University of Ottawa (protocol no. BL-1760). Behavioural experiments were performed under MS-222 (tricaine) anesthesia and all efforts were made to minimize stress on the animals.

### Animals

Wild-type zebrafish (*Danio rerio*) were obtained from a commercial supplier (Mirdo Importations Inc., Montreal, Canada) and held in a closed, re-circulated facility at the University of Ottawa. They were maintained at 28.5°C on a 14:10 hour light:dark cycle. Adults were bred using breeding traps as per methods described by Westerfield [31]. In brief, the resulting embryos were kept in Petri dishes containing E3 embryo medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM  $CaCl_2 \cdot 2H_2O$ , 0.33 mM  $MgSO_4 \cdot 7H_2O$  and 0.3 mg  $l^{-1}$  of methylene blue at pH 7.8) and incubated at 28.5°C for 5 days. At 5 days post fertilization (dpf), larvae were transferred to small tanks filled with treated system water (0.3 mg  $l^{-1}$  with methylene blue).

### Behavioural assays

The larvae used in this study ranged from 14–16 dpf. At this age, zebrafish have fully innervated gill NECs and mature respiratory lamellae [28,32]. Therefore, physiological responses to

neurochemicals can be attributed to branchial rather than extrabranchial (i.e. cutaneous) mechanisms. Larvae were lightly anaesthetized with tricaine (MS-222, Aqualife TMS, Syndel Laboratories, Vancouver, Canada) at 0.04 mg/ml for 2 min and transferred individually into the wells of an opaque, flat-bottomed polystyrene multi-well plate (96-well, Greiner Cellstar, Sigma-Aldrich, Oakville, ON, Canada; [S1 Fig](#), [S2 Fig](#)). Up to 8 larvae were tested during each trial; and each well contained 1 larva. Each well was previously filled with Sylgard (Dow Corning Corporation, Midland, MI, USA) that was left overnight to solidify. This reduced the volume of medium in each well to approximately 100  $\mu$ l and improved observation of fish by reducing movement out of the range of focus of the microscope. Since larvae exhibit a photomotor response when exposed to light during behavioural assays [33], 50  $\mu$ l of 1% methylcellulose and 0.04 mg/ml tricaine was added to each well to minimize movement. Addition of methylcellulose also allowed us to reduce the concentration of MS 222, compared to previous studies [28]. Fish were allowed to adapt to their new environment for 2 min before any observations were made. Normoxic ventilation rate was then obtained and served as a baseline measurement of ventilation frequency ( $f_V$ ). The remaining 50  $\mu$ l of each well was then filled with the treatment drug plus 0.04 mg/ml tricaine or tricaine alone (as the positive control group exposed to hypoxia only). If a stimulatory drug was added,  $f_V$  was measured after an additional 2 min. If an inhibitory drug was applied, the plate was placed in a hypoxic incubator (Forma 3110, ThermoFisher Scientific, Ottawa, ON, Canada) where 100% N<sub>2</sub> was injected to reduce O<sub>2</sub> levels to 1% (8 mmHg).  $P_{O_2}$  was measured and stabilized with a thermal conductivity O<sub>2</sub> sensor. Fish were incubated for 7 min at 28.5°C and  $f_V$  was recorded immediately after. Preliminary experiments had established that an incubation time of 7 min was sufficient to produce hypoxia within the wells and initiate a maximal  $f_V$  response ([S3 Fig](#)). For recovery, each larva was transferred to a new well in normal solution plus 0.04 mg/ml tricaine, and  $f_V$  was recorded after 2 min. For all treatment groups,  $f_V$  was recorded by placing the multi-well plate upon the stage of a stereomicroscope (M6, Leica, Wetzlar, Germany) and capturing 10-s videos using Leica Application Suite. Videos were analyzed *post hoc* and  $f_V$  was calculated. All sample sizes are indicated in the figure legends.

## Drugs

For each drug, a range of concentrations (see [Results](#) and figure legends) was tested in order to determine the concentration that elicited a half-maximal  $f_V$  response (EC<sub>50</sub> value). Serotonin (5-HT) is a major neurotransmitter of gill NECs and was previously shown to increase  $f_V$  in larval zebrafish as early as 7 dpf [23]. 5-HT (cat. no. H9523) was thus used to generate a standard  $f_V$  response and to substantiate the present new procedures for behavioural assay and exogenous drug application. Nicotine (cat. no. N5260), a nicotinic ACh receptor agonist, and atropine (cat. no. A0257), a muscarinic ACh receptor antagonist, were used to target cholinergic receptors. In order to examine the role of purinergic receptors in O<sub>2</sub> sensing, suramin (cat. no. S2671), a broad-spectrum antagonist of P2 receptors, and A-317491 (cat. no. A2979), a novel P2X<sub>3</sub>- and P2X<sub>2/3</sub>-specific antagonist, were used. These drugs were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA). The ATP analogue and purinergic P2 receptor agonist, 2-methylthioadenosine 5'-triphosphate (2-MeSATP), was also tested and was obtained from Tocris Bioscience (cat. no. 1062; Bristol, UK). All drugs were dissolved in water.

## Data analysis and statistics

The D'Agostino-Pearson test for normality was used to determine whether the data collected during behavioural assays conformed to a Gaussian distribution. Repeated measures analysis of variance (ANOVA) was used to compare  $f_V$  between control, treatment and recovery in all

experiments. A Bonferroni *post hoc* test was then used to determine whether mean  $f_V$  was significantly different between control and treatment, and between recovery and control groups ( $P < 0.05$ ).

Curves for dose vs. response were prepared to determine the effective concentration of each drug that gave a half-maximal  $f_V$  response ( $EC_{50}$ ). For excitatory drugs (i.e. 5-HT and nicotine), the response ratio for each individual to each drug concentration was first calculated by dividing treatment  $f_V$  by control  $f_V$ . This procedure was necessary because basal  $f_V$  varied between trials. The response ratio therefore presented a clear measure of the response within each trial relative to control. Means of these ratios from each group were then further normalized to percent of maximum  $f_V$  by the following: maximum  $f_V$  was taken as the highest response ratio within each group, and all other points within that group were divided by this value and converted to percentage. For inhibitory drugs (i.e. suramin and A-317491) that were co-administered with hypoxia,  $f_V$  was suppressed at high concentrations. For these experiments, dose vs. percent maximum inhibition was plotted to calculate  $EC_{50}$ . The response ratio for each drug was calculated as above (i.e. treatment  $f_V$  divided by control  $f_V$ ). Maximum inhibition was taken as the concentration with the lowest response ratio, and this was then divided by other points at each concentration and converted to percentage. For calculation of  $EC_{50}$  in all graphs, a line was fit to the data using a one-phase exponential association model (Prism v5.0, GraphPad Software Inc., La Jolla, CA, USA) with least squares and constrained to a  $y_{max}$  of 100 following the equation  $y = y_{max} (1 - e^{-kx})$ .

## Immunohistochemistry and confocal microscopy

Zebrafish larvae at 16 dpf were prepared for immunohistochemistry following previously established procedures [23,28]. Zebrafish were euthanized with 1 mg ml<sup>-1</sup> MS-222. Whole larvae were then fixed by immersion in a phosphate-buffered solution (PBS; 137 mM NaCl, 15.2 mM Na<sub>2</sub>HPO<sub>4</sub>, 2.7 mM KCl, 1.5 mM KH<sub>2</sub>PO<sub>4</sub> at pH 7.8, [14]) containing 4% paraformaldehyde at 4°C overnight. After rinsing, whole larvae were permeabilized for 24–48 h using 2% Triton X-100 in PBS at 4°C.

Larvae were placed in a solution of primary antibodies, containing polyclonal rabbit anti-5-HT (1:250; cat. no. S5545, Sigma-Aldrich; Antibody ID: AB\_477522) and polyclonal guinea pig anti-P2X3 (1:400–1:1,000; cat. no. GP10108; Neuromics, Edina, MN, USA; Antibody ID: AB\_2283325), and diluted in 0.5% Triton X-100 at 4°C for 24 h. Tissue was then rinsed in PBS and co-treated with secondary antibodies, anti-rabbit fluorescein isothiocyanate (FITC, 1:50; cat. no. 111-095-003, Jackson Immuno Research Laboratories, West Grove, PA, USA) and anti-guinea pig Alexa 594 (1:400; cat. no. A11076, Invitrogen), at room temperature for 1–2 h in the dark. Larvae were rinsed and gill complexes removed and placed on glass slides for imaging.

Polyclonal antibodies for 5-HT were raised in rabbit against a creatinine sulphate complex conjugated with BSA (manufacturer specifications). This antibody has been used to characterize the serotonergic system of gill NECs in several teleost species, including zebrafish [14,23,34]. Antibodies for P2X3 receptors were cloned from rat dorsal root ganglia and recognize residues 383–397 of the carboxy terminus of the receptor. For controls, the P2X3 antibody was pre-treated with a specific blocking peptide (1:100; cat. no. P10108, Neuromics) for 48 h at 4°C before application of the antibody to the tissue for 2 h at room temperature. This procedure eliminated P2X3 immunolabelling.

Tissues stained with fluorescent markers were examined using an upright Nikon A1R microscope with a Nikon Apo 25× water immersion objective lens and a confocal scanning system equipped with Argon (Ar) and Sapphire 561 lasers with peak outputs of 488 nm and 561 nm, respectively. Images were collected using the microscope imaging software NIS Elements

4.13 (Nikon, Tokyo, Japan). Tissues with two fluorescent markers were sequentially scanned with Ar and Sapphire 561 lasers. Images were scanned in optical sections that were 1.5  $\mu\text{m}$  apart for up to 40 sections per z-stack.

## Results

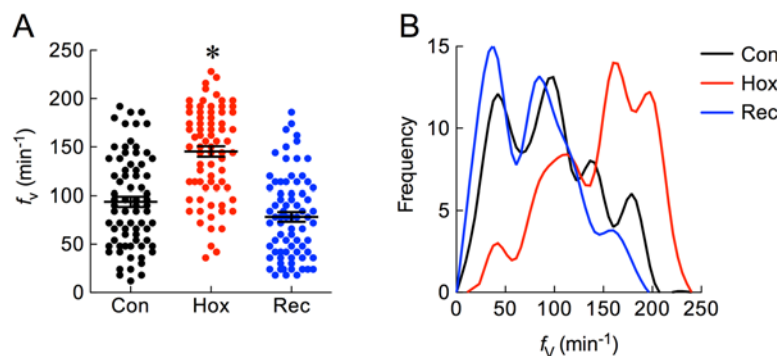
### Verification of procedures for measurement of ventilation frequency following hypoxia or drug treatment

We first established the ventilatory response to hypoxia, in which zebrafish larvae were exposed to a  $P_{\text{O}_2}$  of 8 mmHg for 7 min in multi-well plates. Basal ventilation frequency ( $f_V$ ) in control fish was recorded at  $93.8 \pm 5.4 \text{ min}^{-1}$  and this increased significantly to  $145.3 \pm 5.4 \text{ min}^{-1}$  (a 1.5-fold change) immediately following hypoxic exposure (Fig 1A;  $P < 0.05$ , ANOVA-Bonferroni).  $f_V$  returned to just below basal levels ( $78.2 \pm 5.0 \text{ min}^{-1}$ ) during the recovery period. Tests for normality using D'Agostino-Pearson analysis indicated that data from all 3 groups conformed to a normal distribution (Fig 1B;  $P > 0.05$ ).

Serotonin (5-HT) is a major neurotransmitter in gill NECs and stimulates chemosensory pathways in the gill to increase  $f_V$  in fish [23,35,36]. We used 5-HT in a range of concentrations as a positive control to demonstrate dose-dependent effects of exogenous drug application on  $f_V$ . 5-HT did not have an effect on  $f_V$  at 1  $\mu\text{M}$ , but significantly increased  $f_V$  at 25, 50 and 100  $\mu\text{M}$  (Fig 2A–2D;  $P < 0.05$ , ANOVA-Bonferroni). The response at 50  $\mu\text{M}$  was taken as maximum  $f_V$ , where an  $f_V$  of  $138 \pm 6.9 \text{ min}^{-1}$  was 2.4-fold greater than control. The normalized responses at the concentrations tested were plotted as a percentage of maximum  $f_V$  and indicated an  $\text{EC}_{50}$  of 14.6  $\mu\text{M}$  (Fig 2E).

### Effects of cholinergic drugs on ventilation frequency

Administration of nicotine over a range of concentrations demonstrated a significant dose-dependent effect on  $f_V$ . 1  $\mu\text{M}$  nicotine had only a marginal effect, but at 25, 50, 100 and 200  $\mu\text{M}$  nicotine significantly increased  $f_V$  (Fig 3A–3E;  $P < 0.05$ , ANOVA-Bonferroni). The response at 100  $\mu\text{M}$  was taken as maximum  $f_V$ , where an  $f_V$  of  $169.3 \pm 8.6 \text{ min}^{-1}$  was 2.7-fold greater than control. Note that in these experiments  $f_V$  did not return to basal levels during the recovery period. The normalized responses for nicotine at all concentrations were plotted as a percentage of maximum  $f_V$  and indicated an  $\text{EC}_{50}$  of 24.5  $\mu\text{M}$  (Fig 3F).



**Fig 1. Zebrafish larvae hyperventilated following exposure to severe hypoxia in multi-well plates.** (A) Mean  $\pm$  s.e.m. baseline ventilation frequency ( $f_V$ , in  $\text{min}^{-1}$ ) was first measured in normoxic controls (Con). Application of hypoxia (Hox) increased  $f_V$ , and this was reduced upon recovery (Rec) in normoxic solution. Asterisk indicates a significant difference from control ( $n = 75$ ,  $P < 0.05$ , repeated measures ANOVA-Bonferroni). (B) A frequency histogram of the data from A indicates that the distribution from all 3 groups was Gaussian.

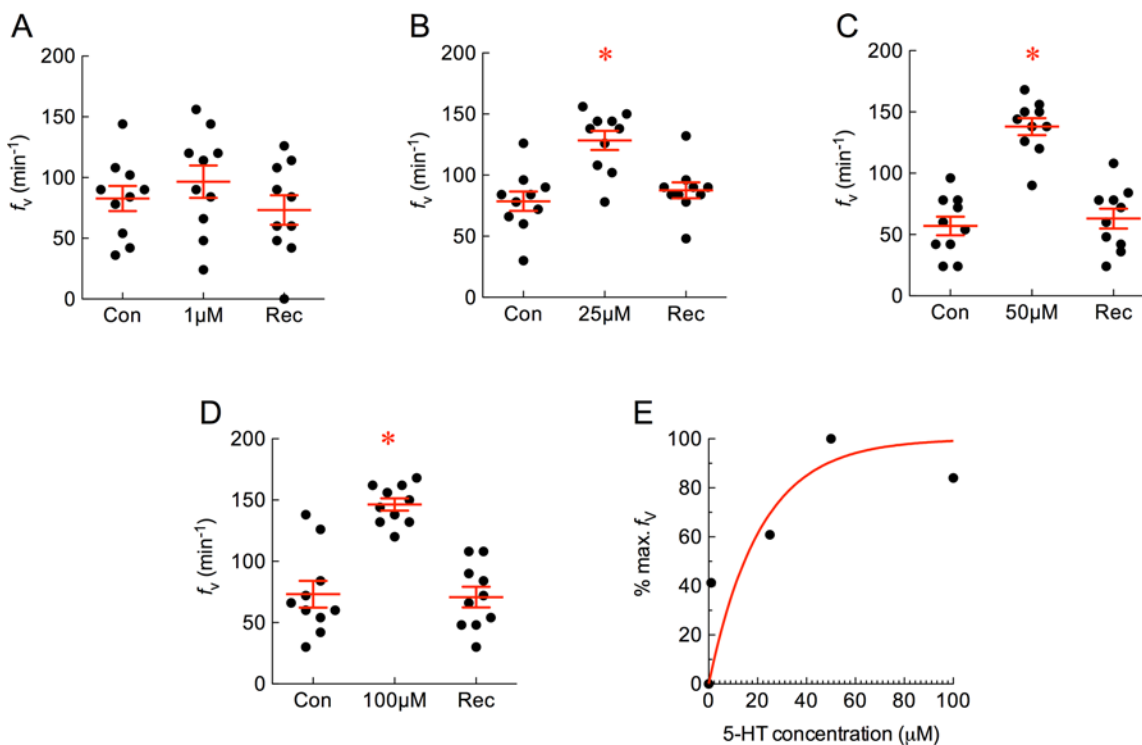
doi:10.1371/journal.pone.0154261.g001

The muscarinic antagonist, atropine, was co-applied with hypoxia but did not inhibit the hyperventilatory response at 1, 25, 50 or 100  $\mu\text{M}$  (Fig 4A–4D).  $f_V$  continued to increase significantly in response to hypoxia ( $P < 0.05$ , ANOVA-Bonferroni) by a factor of 1.4- to 1.5-fold over control values, and this was comparable to the  $f_V$  response to hypoxia alone (Fig 1A). 200  $\mu\text{M}$  atropine, however, abolished the  $f_V$  response to hypoxia (Fig 4E;  $P > 0.05$ , ANOVA-Bonferroni). The results of the atropine experiments are summarized in Fig 4F. Since atropine did not have a significant effect on inhibiting  $f_V$  at most of the concentrations tested, a dose-response model was not generated to estimate  $\text{EC}_{50}$ .

### Effects of purinergic drugs on ventilation frequency

Suramin, a broad-spectrum purinergic antagonist, was co-administered with hypoxia. At 1  $\mu\text{M}$ , suramin had little or no effect, and  $f_V$  increased significantly as a result of hypoxia (Fig 5A;  $P < 0.05$ , ANOVA-Bonferroni). At 25  $\mu\text{M}$ , suramin reduced the hyperventilatory response to hypoxia such that  $f_V$  did not increase significantly above control (Fig 5B;  $P > 0.05$ , ANOVA-Bonferroni). When suramin was applied with hypoxia at 50, 100 and 200  $\mu\text{M}$ ,  $f_V$  was suppressed significantly below basal levels and did not increase during the recovery period (Fig 5C–5E;  $P < 0.05$ , ANOVA-Bonferroni). The effect of suramin at 50  $\mu\text{M}$  was taken as maximum inhibition, where an  $f_V$  of  $40.2 \pm 12 \text{ min}^{-1}$  was 2.1-fold lower than control values. The normalized responses at all concentrations were plotted as a percentage of maximum inhibition and indicated an  $\text{EC}_{50}$  of 30.4  $\mu\text{M}$  (Fig 5F).

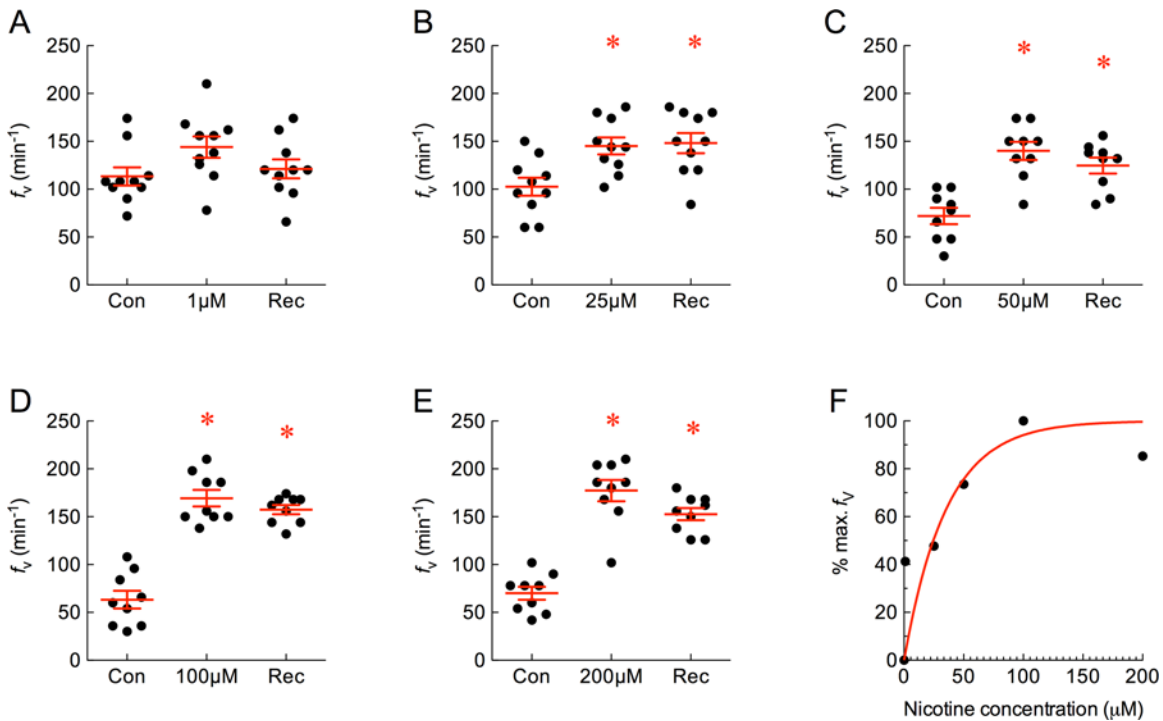
A specific P2X3 blocker, A-317491, was administered with hypoxia. In the presence of the drug at all concentrations ranging from 1–200  $\mu\text{M}$ , hypoxia failed to increase  $f_V$  (Fig 6A–6E;



**Fig 2. Serotonin (5-hydroxytryptamin, 5-HT) increased mean  $\pm$  s.e.m. ventilation frequency ( $f_V$ , in  $\text{min}^{-1}$ ) in a dose-dependent manner.** (A–D) Baseline  $f_V$  was measured as control (Con) and 1, 25, 50 or 100  $\mu\text{M}$  5-HT was applied. Normal solution was replaced during recovery (Rec). Asterisks indicate a significant difference from control ( $n = 10$  for each panel;  $P < 0.05$ , repeated measures ANOVA-Bonferroni). (E) Plot of dose vs. percent of maximum  $f_V$  response. A non-linear one-phase association model with least squares fit indicates an effective half-maximal concentration ( $\text{EC}_{50}$ ) of 14.6  $\mu\text{M}$  for 5-HT.

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**Fig 3. Nicotine increased mean  $\pm$  s.e.m. ventilation frequency ( $f_V$ , in  $\text{min}^{-1}$ ) in a dose-dependent manner.** (A–E) Baseline  $f_V$  was measured as control (Con) and 1, 25, 50, 100 or 200  $\mu\text{M}$  nicotine was applied. Normal solution was replaced during recovery (Rec). Asterisks indicate a significant difference from control ( $n = 10, 10, 9, 9$  and  $9$ ;  $P < 0.05$ , repeated measures ANOVA-Bonferroni). Note that recovery was incomplete at higher concentrations. (F) Plot of dose vs. percent of maximum  $f_V$  response. A non-linear one-phase association model with least squares fit indicates an effective half-maximal concentration ( $\text{EC}_{50}$ ) of 24.5  $\mu\text{M}$  for nicotine.

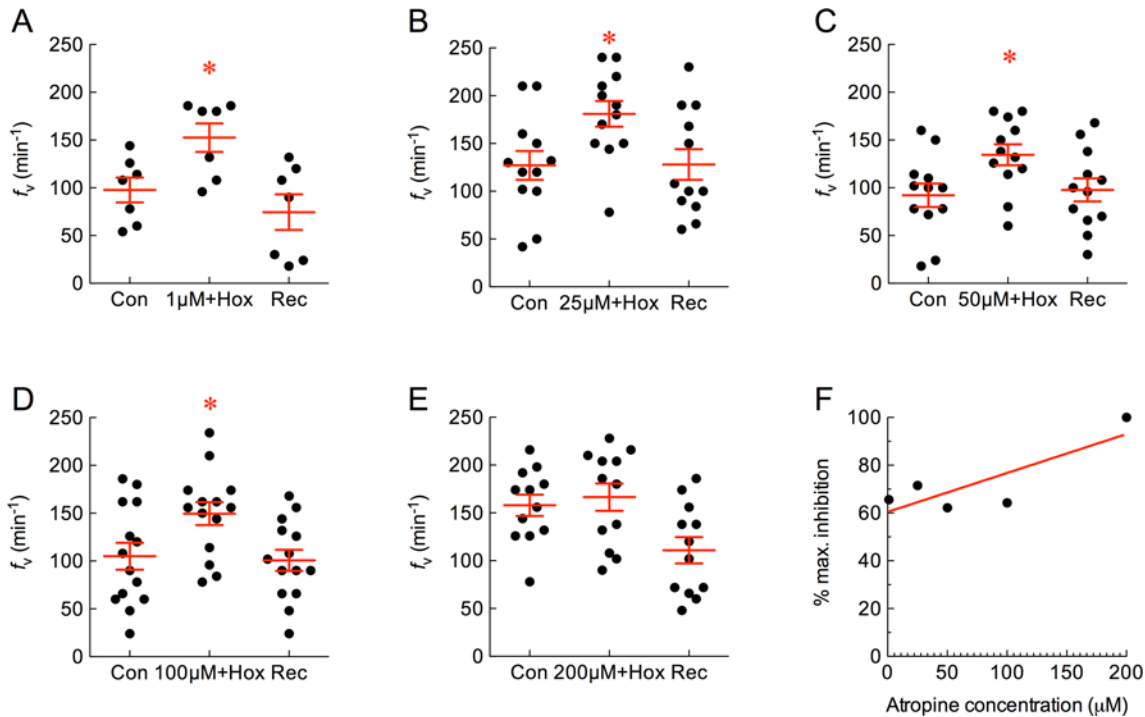
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$P > 0.05$ , ANOVA-Bonferroni). The effect of A-317491 at 50  $\mu\text{M}$  was taken as maximum inhibition, where an  $f_V$  of  $88.8 \pm 15 \text{ min}^{-1}$  was 1.5-fold lower than control values. The normalized responses at all concentrations were plotted as a percentage of maximum inhibition and indicated an  $\text{EC}_{50}$  of 10.8  $\mu\text{M}$  (Fig 6F).

The ATP analogue and P2 agonist, 2-MeSATP, was administered to larvae at concentrations from 1–200  $\mu\text{M}$  (S4 Fig). At 100  $\mu\text{M}$ , 2-MeSATP significantly increased  $f_V$  from  $70.1 \pm 15.4$  to  $116.0 \pm 12.1 \text{ min}^{-1}$ , a factor of 1.6 (S4 Fig;  $P < 0.05$ , ANOVA-Bonferroni). 2-MeSATP produced a marginal increase in  $f_V$  at 50 and 200  $\mu\text{M}$ , but was without effect at lower concentrations.

### Localization of P2X3 receptors

Based on the effects of suramin and A-317491 upon  $f_V$ , we characterized the distribution of P2X3 receptors in the gills using immunohistochemistry (Fig 7). In 21 specimens at 16 dpf, P2X3-immunoreactive cells were found throughout the gill complex. In ventral view, large P2X3 cells were observed in the gill arches among other cell types, such as Merkel-like cells of the gill arches (see [37]) and NECs of the gill filaments, as labelled with anti-5-HT (Fig 7A–7C). Cellular co-localization of P2X3 and 5-HT was not obvious in the tissue from ventral view due to the orientation of the gill filaments, but was clear when gill arches were separated by manipulation and observed at higher magnification (Fig 7D–7F). In the gill arches, P2X3 was localized in 5-HT-positive neurons (Fig 7D and 7E) and was also found in nerve bundles (Fig 7D). In the developing gill filaments P2X3 co-localized with 5-HT in NECs; and P2X3 was also observed in cells and nerve fibres of the filaments that were negative for 5-HT (Fig 7D–7F). Moreover, there were 5-HT-positive NECs that did not express P2X3 (Fig 7D and 7E).



**Fig 4. Atropine attenuated the increase in mean  $\pm$  s.e.m. ventilation frequency ( $f_v$ , in  $\text{min}^{-1}$ ) induced by hypoxia at high concentration.** (A–E) Baseline  $f_v$  was measured as control (Con) and 1, 25, 50, 100 or 200  $\mu\text{M}$  atropine was co-applied with hypoxia. Normal solution was replaced during recovery (Rec). Asterisks indicate a significant difference from control ( $n = 7, 12, 12, 14$  and  $12$ ;  $P < 0.05$ , repeated measures ANOVA-Bonferroni). (F) Plot of dose vs. percent inhibition of maximum  $f_v$ . A linear regression model with least squares fit indicated no significant effect of atropine ( $R^2 = 0.68$ ,  $P > 0.05$ ).

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## Discussion

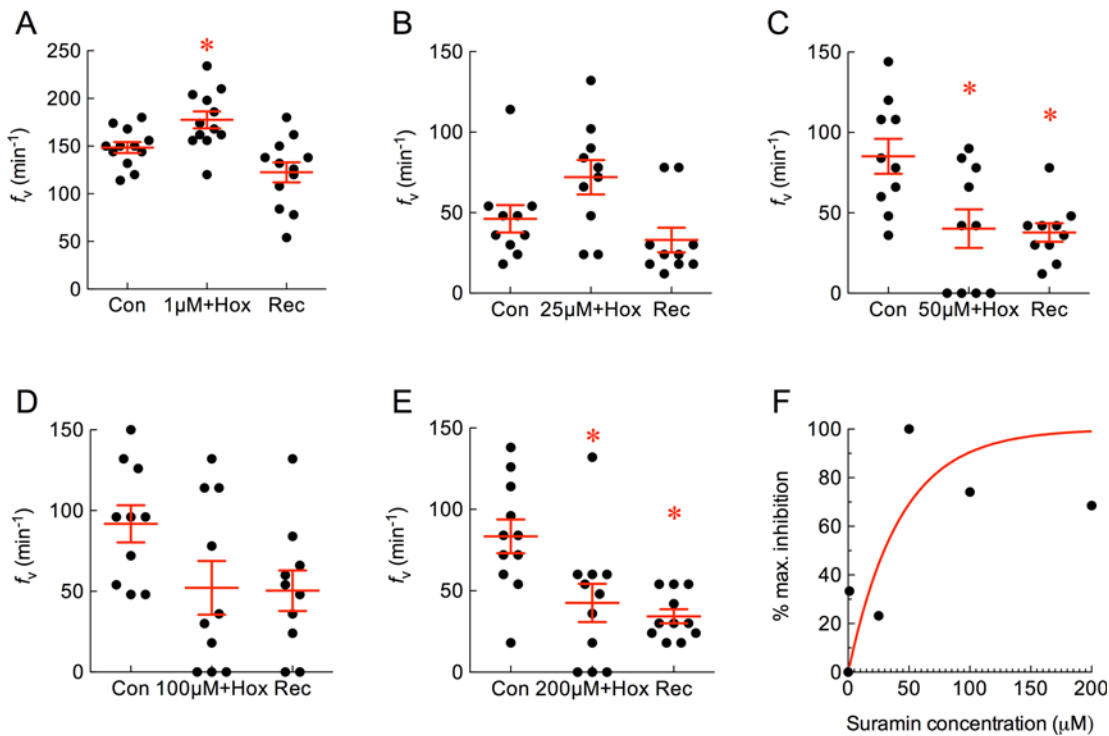
The present study has shown that chemicals with potential effects upon the respiratory response to hypoxia can be rapidly screened in zebrafish at multiple concentrations to generate an activity profile. Our results further demonstrate that purinergic and nicotinic receptors in the gills contribute to the chemosensory response to hypoxia in zebrafish.

## Development of ventilation frequency assays for chemical screening

We have demonstrated a rapid approach to identifying chemical agents that evoke hyperventilation in zebrafish, or that inhibit the hyperventilatory response evoked by co-administration of hypoxia. Previous studies in zebrafish utilized flow-through chambers designed to record the ventilatory response of a single immobilized animal during each trial [23,24,28,38–40]. In the present work, we have made use of multi-well plates to simultaneously treat a greater number of animals, thereby increasing the number of samples per trial. These procedures produced data that conformed to a normal distribution and allowed for parametric statistical testing, rapid generation of dose-response curves for each drug, and calculation of  $\text{EC}_{50}$ . The use of multi-well plates eliminated the need for production of hypoxia by solution exchange within the chamber, which is otherwise time consuming and requires firm immobilization of the specimen to the chamber bottom. Brief placement of multi-well plates in a hypoxic incubator quickly resulted in equilibration of each well to a desired hypoxic  $P_{\text{O}_2}$  within the incubator and produced hypoxic ventilatory rates in zebrafish equivalent to those previously reported at the stages tested [23].

In order to determine how readily exogenously-applied chemicals would diffuse across the gill and affect sensory nerve terminals or NECs, we used 5-HT, an abundant neurotransmitter





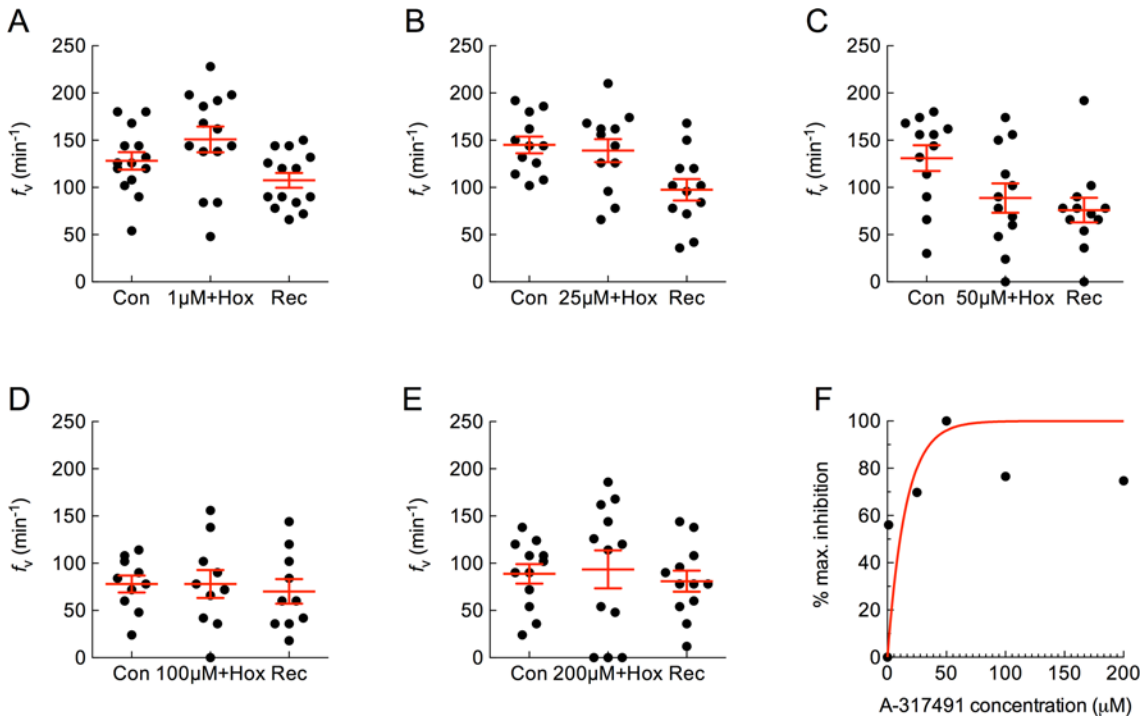
**Fig 5. Suramin attenuated the increase in mean  $\pm$  s.e.m. ventilation frequency ( $f_v$ , in  $\text{min}^{-1}$ ) induced by hypoxia, and suppressed  $f_v$  below basal levels at high concentrations.** (A–E) Baseline  $f_v$  was measured as control (Con) and 1, 25, 50, 100 or 200  $\mu\text{M}$  suramin was co-applied with hypoxia. Normal solution was replaced during recovery (Rec). Asterisks indicate a significant difference from control ( $n = 12, 10, 10, 10$  and  $11$ ;  $P < 0.05$ , repeated measures ANOVA-Bonferroni). Note that in (A) hypoxia increased  $f_v$ , but in other panels suramin suppressed  $f_v$  and at some concentrations  $f_v$  did not recover. (F) Plot of dose vs. percent inhibition of maximum  $f_v$ . A non-linear one-phase association model with least squares fit indicates an effective half-maximal concentration ( $\text{EC}_{50}$ ) of 30.4  $\mu\text{M}$  for suramin.

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in gill NECs known to elicit hyperventilation [13,14,23,35,36], as a positive control. 5-HT increased  $f_v$  in zebrafish at concentrations from 1–100  $\mu\text{M}$  with an  $\text{EC}_{50}$  of 14.6  $\mu\text{M}$ . This concentration range approximately corresponds with mammalian data, where 5-HT elicited responses at 1–50  $\mu\text{M}$  in cells dissociated from the rat carotid body [41,42], and at 5–150  $\mu\text{M}$  in  $\text{O}_2$ -sensitive neuroepithelial bodies in lung slices from neonatal hamster [43]. One limitation to whole-organism chemical screening, however, is that the effects of each compound on post-synaptic terminals or chemoreceptive NECs of the gills may not be easily dissociated from additional non-specific effects outside of the gills, such as on the central nervous system, that may indirectly affect the ventilatory response. However, the blood-brain barrier in zebrafish develops at approximately 3 dpf [44], suggesting that at such low concentrations the effects of exogenously administered chemicals upon the central nervous system may have been limited in our experiments.

### Neurochemical basis of $\text{O}_2$ sensing in the gill

The neurochemical mechanisms that underlie  $\text{O}_2$  sensing in the gill are not yet resolved. NECs, or NEC-like cells, containing 5-HT or a vesicular transporter for ACh have been identified in the gills [13,14,21,23]; however, neither the release of 5-HT nor ACh has been observed during a hypoxic challenge. In zebrafish, recent studies investigating the effects of exogenous drug application *in vivo* have shown that the hyperventilatory response to hypoxia is regulated by metabotropic 5-HT<sub>2</sub> receptors, ionotropic 5-HT<sub>3</sub> receptors, and dopaminergic receptors [23,27]; and that nitric oxide may play a stimulatory or inhibitory role, depending on



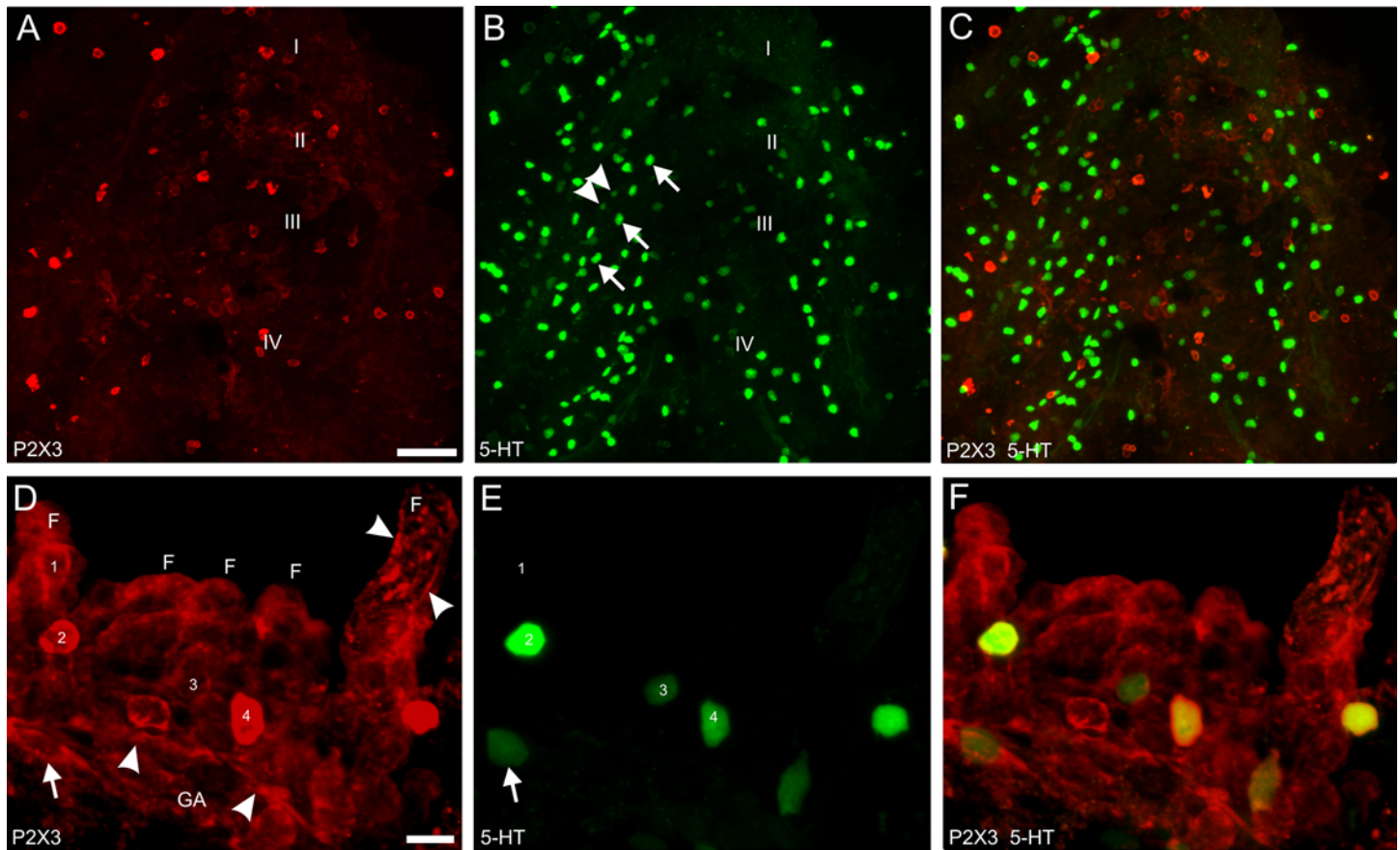
**Fig 6. A-317491 attenuated the increase in mean  $\pm$  s.e.m. ventilation frequency ( $f_v$ , in  $\text{min}^{-1}$ ) induced by hypoxia.** (A–E) Baseline  $f_v$  was measured as control (Con) and 1, 25, 50, 100 or 200  $\mu\text{M}$  A-317491 was co-applied with hypoxia. Normal solution was replaced during recovery (Rec). Treatment groups did not differ from controls ( $n = 14, 12, 12, 10$  and  $12$ ;  $P > 0.05$ , repeated measures ANOVA-Bonferroni). (F) Plot of dose vs. percent inhibition of maximum  $f_v$ . A non-linear one-phase association model with least squares fit indicates an effective half-maximal concentration ( $\text{EC}_{50}$ ) of 10.8  $\mu\text{M}$  for A-317491.

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developmental stage [24]. Earlier studies in the rainbow trout (*Oncorhynchus mykiss*) showed that, compared to ACh, 5-HT induced relatively minor hyperventilation and chemosensory discharge in the gill nerves in the rainbow trout [35,36]. Together these data suggest that hypoxic hyperventilation is regulated by multiple excitatory, inhibitory or modulatory neurotransmitters in the gill.

In type I cells of the mammalian carotid body, the homologues of gill NECs, the primary excitatory neurotransmitters that activate afferent nerve terminals in the carotid body include ACh and ATP [6–11]. In trout, ACh and nicotine produced pronounced sensory discharge in the gill and hyperventilation [35,36]; whilst muscarine and atropine had only minor effects. In zebrafish, preliminary evidence demonstrated that application of the cholinergic agonist, nicotine, increased  $f_v$  [27]; whilst the nicotinic antagonist, hexamethonium, inhibited the hyperventilatory response to hypoxia [23]. The present work agrees with these findings and found that nicotine produced a significant and dose-dependent elevation of  $f_v$ ; and that atropine was without significant effect, inhibiting the hypoxic response only at high concentration.

Previous studies had not tested whether purinergic drugs might affect  $f_v$  in fish. In the present study, we used suramin, a broad-spectrum antagonist of purinergic P2 receptors, as well as an additional antagonist, A-317491, with specificity for P2X3 and P2X2/3 receptors. Both drugs showed concentration-dependent effects, eliminated the hyperventilatory response to hypoxia, and further reduced  $f_v$  below resting levels. Moreover, A-317491 had a lower  $\text{EC}_{50}$  than suramin. Administration of the ATP analogue and P2 agonist, 2-MeATP, had limited effects across a range of concentrations, perhaps due to poor diffusion of this drug across the gill epithelium, but successfully induced hyperventilation at 100  $\mu\text{M}$ . It was thus apparent from



**Fig 7. Immunohistochemical localization of P2X3 receptors in the zebrafish gill at 16 days post-fertilization.** Co-labelling with antibodies against P2X3 (red) and serotonin (5-HT, green) are shown at low magnification in a whole gill complex in ventral view (A–C), and at higher magnification in an isolated gill arch (D–F). (A) Many P2X3-positive cells were observed on all four gill arches, as indicated by numerals I–IV on the left side of the gill complex. (B) Labelling by anti-5-HT identified primarily Merkel-like cells (arrows) of the gill arches with few neuroepithelial cells (NECs, arrowheads) of the gill filaments visible. (C) Merge of panels A and B. (D) In isolated gills, the gill arch (GA) displayed P2X3-positive neurons (arrow), and a nerve bundle (arrowheads); gill filament primordia (F) retain several P2X3-positive cells (numbered 1–4; note that cell 3 is P2X3-negative). In addition, the filament at the right displayed a pattern of P2X3 activity that was reminiscent of nerve fibres (arrowhead). (E) 5-HT immunoreactivity revealed that P2X3-positive neurons were also 5-HT positive. From D, cell 1 was 5-HT-negative; cells 2 and 4 were P2X3- and 5-HT-positive; cell 3 was 5-HT-positive and P2X3-negative. (F) Merge of panels D and E. Scale bar in A = 50  $\mu$ m; in B = 10  $\mu$ m.

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our experiments that endogenous release of ATP, as during hypoxia, was more effective in producing hyperventilation than exogenous application of the ATP analogue. We confirmed these observations of purinergic activity from behavioural assays by localizing P2X3 receptors to the gills of zebrafish larvae using immunohistochemistry. A previous study found P2X3 labelling in the gill epithelium of adult zebrafish [14]. In the present study, we determined that P2X3 receptors were found in serotonergic neurons of the gill arches. In adult zebrafish, serotonergic neurons reside in the proximal aspect of each gill filament and extend axons that innervate chemoreceptive NECs [14]. In the developing gills of larvae, however, these neurons are observed in the gill arches and appear to migrate to the base of the filaments as the gill matures and increases in size [28]. We suggest that P2X3/5-HT-positive neurons of the gill arches in larvae innervate the NECs of the filaments and provide an afferent pathway for initiation of autonomic reflexes, such as hyperventilation, during hypoxic stimulation of NECs. Indeed, in the present study P2X3 also labelled nerve bundles of the gill arch and generated a labelling pattern in the gill filaments that resembled that of innervation in developing zebrafish [28].

Our present and previous studies [23] suggest a role for ACh via nicotinic receptors, and ATP via P2X3 and possibly other purinergic receptors, as potential neurotransmitters in gill O<sub>2</sub> sensing in zebrafish. Most compelling among these data is that the hyperventilatory response to hypoxia was inhibited by antagonists of nicotinic receptors [23] and of purinergic receptors, perhaps due to the endogenous release of ACh and ATP, respectively, elicited by hypoxia. These results are in line with evidence from the mammalian carotid body, where both nicotinic and P2X2/3 receptors are found at post-synaptic terminals of petrosal neurons and play critical roles in chemoexcitation during hypoxia [6,8,9–11,45]. We also localized P2X3 activity within 5-HT-positive NECs of the filaments, as well as in other non-serotonergic cells. These observations may indicate a paracrine role for P2X3 receptors within NECs in modulating neurotransmitter release in the gill. For example, in the carotid body "ATP-induced ATP release" involving purinergic P2Y2-expressing glial-like cells can increase the excitatory ATP signal at the chemosensory synapse [46]. In addition, P2X3 expression in NECs may form part of an autocrine, or positive feedback, pathway in which ATP release may be further potentiated. Such a feedback mechanism involving P2X receptors has been observed in taste receptor cells [47]; and P2X2 receptors were localized to carotid body type I cells in rat following exposure to chronic hypoxia [48].

## Conclusions and applications

In this article, we have presented evidence for the novel action of purinergic drugs upon the ventilatory response to acute hypoxia in zebrafish. Our studies suggest that a role for ACh and ATP as neurotransmitters in peripheral O<sub>2</sub> chemosensing may not be limited to the carotid body in mammals. Furthermore, such a mechanism may have arisen earlier during vertebrate evolution—before the appearance of air-breathing vertebrates. The way in which ACh and ATP may work with 5-HT in the fish gill to coordinate the hypoxic response is unclear, but may involve neurotransmitter release from multiple chemoreceptor populations, and a combination of synaptic, paracrine and autocrine pathways. Exploiting the ventilatory response in zebrafish for chemical screening will help create a neurochemical profile of control of the hypoxic response in this model organism, and may lead to the identification of novel drugs that affect peripheral O<sub>2</sub>-sensing chemoreceptors. By extension, such drugs may be tested subsequently in mammalian systems and developed as therapeutic agents for human disorders involving the carotid body, such as hypertension, chronic heart failure, obstructive sleep apnea, sudden infant death syndrome, and diabetes [5,49,50].

Additionally, results of these studies may contribute to the establishment of new policies aimed at the preservation of water ecosystems. Effluent from industry, pharmaceuticals and household products may have profound effects upon the health of aquatic organisms, including fish. Ammonia, a commercially important chemical, acts directly upon gill NECs and causes hyperventilation in fish [25,51]. Fluoxetine, the selective 5-HT reuptake inhibitor in the antidepressant, Prozac, is found in effluent [52] and is a ventilatory stimulant in rainbow trout [53]. A laboratory screen for the ventilatory effects of chemicals isolated from the environment may establish a procedure for early detection of chemicals that may otherwise have catastrophic effects upon aquatic populations.

## Supporting Information

**S1 Fig. Illustration of a multi-well plate used in behavioural assays.** The first three rows (rows A, B and C) are coated with Sylgard (see [Materials and Methods](#)). The well marked "A1" is additionally filled with solution and a zebrafish larva. Scale bar = 16 mm. (TIF)

**S2 Fig. Illustration of a single larva immersed in solution in a well of a multi-well plate.**

The image is the same as in well "A1" in [S1 Fig](#) but at higher magnification. The well was previously coated with Sylgard and contains solution. Scale bar = 2 mm.

(TIF)

**S3 Fig. Preliminary trials determined the optimal duration for exposure of zebrafish larvae to 8 mmHg hypoxia.**

Mean  $\pm$  s.e.m. ventilation frequency ( $f_V$ , in  $\text{min}^{-1}$ ) was measured in normoxic controls (Con) and after application of hypoxia (Hox) for 5 min (A), 7 min (B), and 10 min (C). Asterisks indicate a significant difference from control ( $n = 10, 7, \text{ and } 8$  in panels A–C;  $P < 0.05$ , paired  $t$ -test). (D) Data taken from the previous panels indicated that 7 min hypoxia produced the greatest increase in  $f_V$  above controls.

(TIFF)

**S4 Fig. 2-MeSATP increased mean  $\pm$  s.e.m. ventilation frequency ( $f_V$ , in  $\text{min}^{-1}$ ).** (A–E) Baseline  $f_V$  was measured as control (Con) and 1, 25, 50, 100 or 200  $\mu\text{M}$  2-MeSATP was applied. Normal solution was replaced during recovery (Rec). Asterisk in D indicates a significant difference from control ( $n = 11, 10, 10, 9$  and 10;  $P < 0.05$ , repeated measures ANOVA-Bonferroni).

(TIFF)

## Author Contributions

Conceived and designed the experiments: SR WP MGJ. Performed the experiments: SR WP. Analyzed the data: SR WP MGJ. Wrote the paper: SR WP MGJ.

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